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Toxic Effects of Single-Walled Carbon Nanotubes in the Development of *E. coli* Biofilm

DEBORA F. RODRIGUES AND MENACHEM ELIMELECH*

Department of Chemical Engineering, Environmental Engineering Program, Yale University, New Haven, Connecticut 06520-8286

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The impact of single-walled carbon nanotubes (SWNTs) on the different developmental stages of biofilms has been investigated using *E. coli* K12 as a model organism. Specifically, we investigated (i) the impact of SWNT concentration on cell growth and biofilm formation, (ii) toxic effects of SWNTs on mature biofilms, and (iii) formation of biofilm on SWNT-coated surfaces. The results show that at the initial stage of biofilm formation, SWNTs come into contact with bacterial cells prior to biofilm maturation and inhibit their growth. Furthermore, the results suggest that bacteria in mature biofilms are less sensitive to the presence of SWNTs than cells in other biofilm stages, similar to previous observations of biofilm resistance to antimicrobials. In mature biofilms, the soluble exopolymeric substances (EPS) secreted by the biofilm play an important role in mitigating the toxic effects of SWNTs. Upon exposure to SWNTs, biofilms without soluble EPS in the supernatant had a much more significant loss of biomass because of cell detachment from the biofilm than biofilms containing soluble EPS. To observe similar cell loss, biofilms with soluble EPS needed SWNT concentrations that were 10 times higher compared to biofilms without soluble EPS. Finally, SWNTs deposited onto surfaces affected significantly the subsequent biofilm development. Analysis of the total biomass and the area occupied by cells indicates that a SWNT-coated substratum has 10 times less biofilm colonization and biomass production than a control substratum without SWNTs.

Introduction

There has been much debate in recent years on the environmental implications of nanomaterials. Nanomaterials have the potential for wide-ranging applications in medicine, electronics, and energy production. Carbon nanotubes (CNTs) are among the most promising engineered nanomaterials because of their unusual physicochemical, mechanical, and electrical properties (*1*). CNTs are already being used in many applications, ranging from consumer electronics to drug delivery (*2*). Because of potential new applications and the likelihood of large-scale production, it is likely that CNTs will find their way into aquatic environments, resulting in adverse ecotoxicological impacts.

In most natural and engineered aquatic systems, planktonic bacteria tend to colonize surfaces by forming microbial consortia called biofilms (*3*). Biofilm formation begins with the adhesion of single cells to the substratum, followed by cell proliferation and exopolymeric substances (EPS) production (*4*). These sessile communities can have a number of beneficial applications, such as biocontrol agents by preventing infections in certain plants (*5*), in situ remediation (*6*), and breakdown of organics in wastewater treatment plants (*7, 8*). However, biofilms can also have deleterious effects, causing antibiotic-resistant infections (*9, 10*), clogging of pipes (*11, 12*), and contaminating food in industrial settings (*13*). Given the ubiquity of biofilms in aquatic environments, as well as their medical and economic impacts, there is a critical need to understand these communities and the potential toxic effects of CNTs on their development.

Although recent studies have shown that CNTs are toxic to a variety of microorganisms (*14–16*), research addressing the impact of CNTs on microorganisms in biofilms has not yet been explored. Previous studies have shown that several microorganisms are sensitive to antibiotics and toxic compounds when in the planktonic stage or at the initial biofilm stage (transition from motile to sessile stage), while they exhibit resistance to toxic substances in fully mature biofilms (*17, 18*). These findings suggest that toxic substances, such as CNTs, can have different toxic effects on fully mature biofilms than they do on other cell stages. Consequently, it is imperative to understand the microbial response of biofilms to CNT exposure.

The study of toxic effects of CNTs on biofilms will provide valuable insights into the potential impact of these emerging engineered nanomaterials on microorganisms in aquatic systems. CNTs could interact with microorganisms in aquatic environments in three possible scenarios. First, CNTs could interact with bacterial cells prior to biofilm formation and inhibit their growth and biofilm formation. Second, CNTs could disturb the mature biofilm leading to its detachment; in this case, the EPS secreted by the biofilm could also play an important role as a barrier against the negative effects of CNTs. Finally, CNTs could deposit onto surfaces in aquatic environments, such as sediments and rocks, thereby impacting subsequent biofilm formation.

The overall objective of this paper is to study the effects of single-walled carbon nanotubes (SWNTs) on bacterial (*Escherichia coli* K12) growth and biofilm formation under conditions that may simulate potential scenarios of SWNT release into aquatic systems. Specific objectives include the investigation of (i) the impact of SWNT concentration on cell growth and biofilm maturation, (ii) the toxic effects of SWNTs on mature biofilms, and (iii) the formation of biofilm on SWNT-coated surfaces. Our results show that cell growth and biofilm formation are dependent on SWNT concentration, with higher concentrations leading to greater inhibition of bacterial growth and biofilm formation. Furthermore, surfaces coated with SWNTs significantly inhibit biofilm formation.

Materials and Methods

Single-Walled Carbon Nanotubes (SWNTs). The pristine SWNTs used in this study were purchased from Stanford Materials (SWNT-90, lot #082106). The preparation and characterization techniques as well as the SWNT physicochemical properties are described in our previous publications (*14, 19*). Briefly, the SWNTs had an average outer diameter of 1.2 nm, lengths in the range of 10 to 20 μm, and a specific surface area of 407 m^2/g. Raman spectra (532 nm) and thermogravimetric analysis (TGA) showed that the

* Corresponding author e-mail: menachem.elimelech@yale.edu; phone: (203) 432-7195.

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SWNTs were highly ordered (G/D band ratio of 31), not functionalized, and contained 6% (w/w) metals.

Bacterial Strains and Culture Conditions. The *E. coli* K12 MG1655 used in this study was obtained from the Genome Center at the University of Wisconsin (*20*). We used a minimal M63 medium (*21*) supplemented with 1% D-mannose as a carbon source. Cells were grown at 37 °C in all experiments, unless indicated otherwise. To prepare the frozen stocks, cells were grown overnight at 37 °C. Sterile glycerol was added to the cell suspensions (25% final concentration), and the mixture was distributed aseptically in 1 mL aliquots in 1.5 mL cryogenic tubes and stored at −80 °C. For each experiment, fresh plates of M63 agar medium supplemented with 1% D-mannose were grown overnight at 37 °C from the frozen stocks. A colony was then transferred to sterile M63 broth, and again was grown overnight before each experiment. The overnight culture at a final cell concentration of 5×10^8 CFU/mL was diluted (1:100) for all experiments.

Cell Growth and Biofilm Development Assays in Suspended SWNTs. Cell growth and biofilm development in the presence of different concentrations of suspended SWNTs were monitored with sterile, non-treated 96-well flat-bottom microtiter plates (BD Falcon). Both the SWNTs and the M63 media with 1% D-mannose were autoclave-sterilized. Before each experiment, the SWNTs were dispersed in the media by probe sonication for 30 min (Misonix 3000, Misonix Inc., Farmingdale, NY). The tip of the sonicator was carefully cleaned with 70% ethanol and rinsed three times with sterile distilled water before and after use. After sonication, we performed a serial dilution in M63 supplemented with 1% D-mannose to obtain solutions with different concentrations of SWNTs (0, 0.5, 1, 5, 10, 20, 30, 40, 50, 70, 90, 100, 110, 130, 150, 200, 250, 300, 350, 400, 450, and 500 mg/L). We added 300 μL of each SWNT concentration to 12 wells of the 96-well microtiter plates. An overnight *E. coli* K12 culture (3 μL) was then added to inoculate the bacteria (dilution ratio of 1:100) in 6 of the 12 wells, with the other 6 wells being used as blanks (no bacteria were inoculated in the media with SWNTs) for each SWNT concentration. All the plates were incubated for 48 h at 37 °C.

The total cell growth in the presence of different concentrations of suspended SWNTs was measured prior to the biofilm measurements. Briefly, after 48 h of incubation at 37 °C, the microtiter plates were measured at OD_{600} with a microplate reader (SpectraMax 340PC, Molecular Devices). The readings from wells without bacteria (blanks) were averaged out and subtracted from the readings from wells with *E. coli* K12. Note that the total cell growth represents the cells in the supernatant and the biofilm on the wall of the microtiter plates.

The quantification of cell concentration (or biomass) in biofilms was carried out as described elsewhere (*22, 23*). Briefly, after each cell growth assay, the cells in the microtiter plates were washed 3 times with phosphate buffered saline (PBS) solution (1 mM KH_2PO_4, 1 mM Na_2HPO_4, 2.7 mM KCl, and 13.7 mM NaCl at pH 7.4) to remove suspended, unbound cells. The cells in the remaining biofilm were then stained for 20 min with 300 μL of 0.1% crystal violet solution and washed again with the PBS solution. Cells bound to the walls of the microtiter plates were quantified by adding 300 μL of acetone/ethanol (20:80, v/v) and measuring the OD_{540} with a microplate reader (SpectraMax 340PC, Molecular Devices). The OD_{540} readings from wells without bacteria (blanks) were averaged out and subtracted from the readings from wells with bacteria. The standard deviations were also calculated for each condition.

Biofilm Formation in the Presence of Dead Cells. Biofilm development in the presence of different concentrations of dead cells was monitored with sterile, non-treated 96-well flat-bottom microtiter plates. The purpose of these experiments was to better interpret the results of cell growth and biofilm formation in the presence of SWNTs. Specifically, these experiments test the hypothesis that live cells can utilize dead cells as a carbon source and enhance cell growth and biofilm development. The cells were prepared by growing 15 mL of *E. coli* K12 overnight in M63 with 1% D-mannose. The cells were washed 3 times with PBS by centrifugation at 6,000 $\times$ *g* for 10 min and resuspended in 1.5 mL of M63 media without D-mannose. The number of cells resuspended in M63 was quantified by the plate count method. The rest of the cells (1.3 mL) were autoclaved to inactivate the cells. A sterility test, to ensure that all cells were dead, was performed in triplicate by plating 50 μL of autoclaved cells on LB plates. A serial dilution (10°, 10^{-1}, 10^{-3}, 10^{-5}, 10^{-7}) of the 5×10^8 CFU/mL dead cells was performed in M63 supplemented with 1% D-mannose to obtain different concentrations of dead cells for the cell growth and biofilm assay. We added 300 μL of each dead cell dilution to 12 wells of the 96-well microtiter plates. An overnight *E. coli* K12 culture (3 μL) was then inoculated in 6 of the 12 wells (i.e., dilution ratio of 1:100); the other 6 wells were used as blanks (i.e., dead cells and M63 media supplemented with 1% D-mannose). For each dead cell concentration, the total cell growth and the biofilm cell concentration were measured after 48 h of growth at 37 °C using the same procedures described in the previous subsection.

Exposure of Mature Biofilm to SWNTs with or without Soluble EPS. The effect of different concentrations of SWNTs on a mature biofilm in the presence or absence of soluble EPS in the media was monitored with sterile, non-treated 96-well flat-bottom microtiter plates. The experiments involved the following five steps: (i) production of soluble EPS, (ii) preparation of SWNTs suspended in soluble EPS, (iii) preparation of mature biofilm for experiments with and without EPS, (iv) quantification of the impact of SWNTs on mature biofilm in the presence of soluble EPS, and (v) quantification of the impact of SWNTs on mature biofilm without soluble EPS.

To obtain a significant amount of soluble EPS, a flask with 50 mL of M63 media supplemented with 1% D-mannose was inoculated with *E. coli* K12 and incubated at 37 °C without shaking for 48 h. Next, the cells from the flask were centrifuged at 6,000 $\times$ *g* for 10 min, and the supernatant was filtered with a 0.22 μm low protein binding Millex membrane (Millipore, Billerica, MA) to ensure that all cells were completely removed from the supernatant, leaving only the secreted soluble EPS (330 $\pm$ 10 mg/L TOC).

To prepare SWNT suspensions for the experiments, we added 500 mg/L SWNTs to an aliquot of filtered soluble EPS and sonicated for 30 min to disperse the SWNTs. Following this step, the filtered EPS was used as a stock solution for a serial dilution of the dispersed SWNTs to obtain different concentrations of SWNTs (1, 5, 10, 20, 40, 70, 90, 100, 130, 150, 200, 300, 400, and 500 mg/L). A control containing only EPS (i.e., no SWNTs) was also prepared.

To prepare mature biofilms, the first 6 columns of the microtiter plate (48 wells) were inoculated with *E. coli* K12 in M63 media supplemented with 1% D-mannose and grown at 37 °C without shaking for 48 h to obtain a mature biofilm. The other 6 columns of each plate contained just the media without the bacteria, to serve as blanks.

For biofilm assays in the presence of soluble EPS, the media from the mature biofilm and the corresponding blanks were gently poured out to remove the supernatant and to retain the biofilm attached to the microtiter plate. Each different concentration of SWNTs with EPS and the controls containing soluble EPS without SWNTs were added to one row (12 wells) of the 96-well microtiter plates to have 6 replicates of biofilm exposed to SWNTs with EPS and blanks

of SWNTs with EPS (i.e., no biofilm). One of the rows of each plate had EPS without SWNTs as a control. All the microtiter plates were incubated for 1 h in the dark at 37 °C, and quantification of the final concentration of cells in the biofilm was measured by the biofilm assay described earlier in the subsection Cell Growth and Biofilm Development Assays in Suspended SWNTs.

For biofilm assays without soluble EPS, the media from the mature biofilm and the corresponding blanks were gently poured out and rinsed 4 times with M63 media supplemented with 1% D-mannose to remove soluble EPS secreted by the biofilm. The same SWNT concentrations were prepared in M63 media with 1% D-mannose but without soluble EPS. The SWNTs in the M63 media with 1% D-mannose were distributed into the plates and incubated for 1 h in the dark at 37 °C. This assay contained a control of M63 media with 1% D-mannose without SWNTs in one row of the microtiter plate.

Procedure for Coating Slide Surfaces with SWNTs. Circular coverslips, 12 mm in diameter, were cleaned overnight at 75 °C with 2% Hellmanex II solution and then rinsed with deionized water. The clean coverslips were spin-coated (Spin Coater model SCS P-6708, Specialty Coating Systems Inc., Indianapolis, IN) with 80 μL of 2% (w/v) poly(DL-lactide-co-glycolide) (PLGA) dissolved in chloroform. For a control (no SWNTs), coverslips were coated with PLGA in a spin coater at 300 rpm for 70 s and were allowed to completely dry for 3 days before the experiment. To coat the coverslips with SWNTs, 6 mg of SWNTs was suspended in 100 mL of ethanol. The suspension was sonicated for 10 min to disperse the SWNTs. Then, the mixture was filtered through a 5 μm Omnipore PTFE membrane (Millipore, MA) and dried under vacuum for 30 min to form a homogeneous SWNT coating on the membrane. After coating the clean coverslips with PLGA in the spin coater at 300 rpm for 50 s, the side of the membrane containing the SWNTs was gently pressed against three freshly coated PLGA coverslips to "glue" the SWNTs. Following 3 days of drying at room temperature, the membrane containing the SWNTs attached to the coverslips was carefully cut with a hole-punch measuring 13 mm in diameter. The membrane was then peeled off with forceps, leaving SWNT-coated coverslips. Both the control and SWNT-coated coverslips were sterilized by UV for 15 min.

Biofilm Assay on Immobilized SWNTs. To examine biofilm formation on the coverslips (control and SWNT-coated), the coverslips were deposited at the bottom of 24-well flat-bottom microtiter plates (BD Falcon). *E. coli* K12 was then allowed to grow for 48 h at 37 °C under static conditions in M63 media supplemented with 1% D-mannose. After the incubation period, the coverslips were removed from the bottom of the plates and washed twice gently with M63 media only. The coverslips were then stained using the LIVE/DEAD BacLight Bacterial Viability Kit (Invitrogen Corp., Carlsbad, CA) with propidium iodide (PI) and SYTO9 for probing dead and live cells, respectively, as per manufacturer's instructions. Both controls and the SWNT-coated coverslips were done in triplicates.

Confocal Microscopy and Image Analyses. Microscopic observation and image acquisition of the biofilms on the slides were performed using a Bio-Rad MRC-1024 Krypton/Argon laser scanner mounted to a 2FL reflector slider on a Zeiss Axiovert equipped with DIC optics (PlanApo ×10, Carl Zeiss). In all experiments, three stack images were acquired from random positions on each slide. Images were acquired at 2 μm intervals down through the biofilm, with the number of images in each stack changing according to the thickness of the biofilm. Laser scanning confocal microscope (LSCM) images were generated using the BioRad confocal assistant software (version 4.02). Biofilm thickness was determined by multiplying the number of slices taken in the biofilm by the thickness of each slice (2 μm). Each confocal image stack was saved as a series of tiff images and later converted into eight-bit grayscale images using ImageJ software (NIH) for analyses in COMSTAT (*24*). COMSTAT was then used to threshold the images to reduce background noise. For each image stack, total biomass ($\mu m^3/\mu m^2$), the percentage of area occupied by cells, the average and maximum thickness, and the roughness coefficient, as an indicator of biofilm heterogeneity, were calculated by COMSTAT from the threshold images.

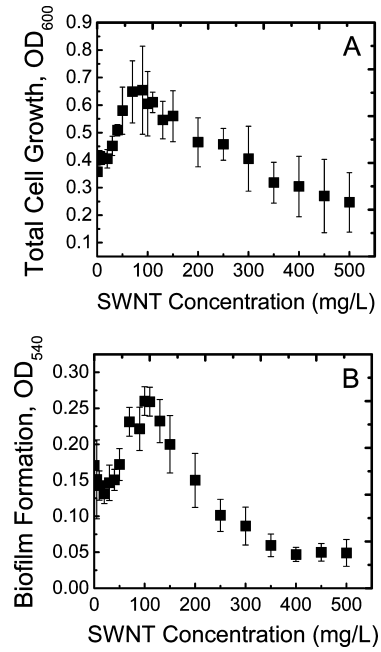

FIGURE 1. Effects of different SWNT concentrations on *E. coli* K12: (A) total cell growth corresponding to the OD_{600} of both suspended and biofilm growth in the microtiter plate, and (B) biofilm formation in the microtiter plate after 48 h. Experiments were conducted under static conditions at 37 °C in M63 media supplemented with 1% D-mannose. Each data point represents the average optical density measurements from 6 replicates grown for 48 h in the presence of different concentrations of SWNTs. Error bars represent one standard deviation.

Results and Discussion

Cell Growth and Biofilm Development in Presence of Suspended SWNTs. Although CNTs appear to be toxic to a variety of microorganisms (*14, 16, 25*), to date no studies have addressed the impact of CNTs on complex microbial structures, namely, biofilms. Furthermore, most previous studies exposed microbes to nanomaterials for only a short period of time (only a few hours) (*14, 25*), leading to the question of whether microbes in aquatic systems can recover with time from the toxic effects of these nanomaterials and form biofilms.

Our results show that both total cell growth and biofilm formation of *E. coli* were enhanced after 48 h of exposure to SWNTs at concentrations varying from 5 to ~300 mg/L (Figure 1), with a maximum cell growth and biofilm enhancement at 100 mg/L. However, exposures at higher SWNT concentrations (>300 mg/L) resulted in increased inhibition of both total cell growth and biofilm formation. The enhancement of cell growth and biofilm formation at SWNT concentrations ranging from 5 to ~300 mg/L indicates that biofilms in aquatic systems can potentially recover from the toxic effects of SWNTs after long exposure times (48 h) at low to moderate concentrations of SWNTs, but not at higher SWNT concentrations.

Previous studies with bacteria exposed to CNT concentrations ranging from 5 to 20 mg/L for short periods of time

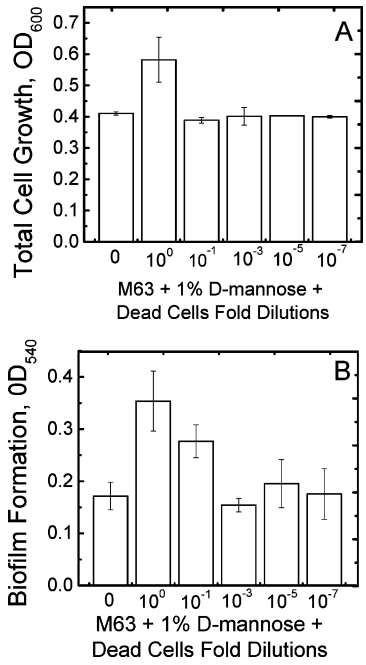

FIGURE 2. Effects of different concentrations of dead cells on *E. coli* K12: (A) total cell growth corresponding to the OD_{600} of both suspended and biofilm growth in the microtiter plate, and (B) biofilm formation in the microtiter plate after 48 h. Experiments were conducted under static conditions at 37 °C in M63 media with 1% D-mannose supplemented with different dilutions of dead cells (starting with 5 × 10^8 CFU/mL at 10^0 dilution). Each data point represents the average optical density measurements from 6 replicates grown for 48 h in the presence of different concentrations of dead cells. Error bars represent one standard deviation.

(less than 1 h) demonstrated that CNTs are toxic to bacteria (*14, 25*). Our results suggest that SWNT toxicity to growing bacterial cells at lower to moderate concentrations (i.e., between 5 and 300 mg/L) may occur only temporarily since there is still cell growth at these concentrations and even enhancement of biofilm production and suspended cell growth (Figure 1). The increased inhibition of both total cell growth and biofilm formation at higher concentrations of SWNTs, with a maximum inhibition at 500 mg/L, can be explained by the fact that the cell concentrations in the experiments are kept constant, but the amount of SWNTs is increased systematically. Eventually, the SWNTs added to the media reach a concentration high enough to inactivate all the cells initially inoculated in the media, thereby preventing the cells from recovering to their normal growth and biofilm formation, even after 48 h of incubation. All toxic compounds have a threshold tolerated by microorganisms (*26*), and in the case of *E. coli*, under the conditions examined, SWNT concentrations >300 mg/L lead to inhibition of growth and biofilm formation.

Enhancement of Cell Growth and Biofilm Formation in Presence of Dead Cells. Previous studies have shown that SWNTs are cytotoxic to bacteria by damaging the cell wall, resulting in release of intracellular materials, such as DNA and RNA (*14*). These released intracellular materials and others (e.g., lipids, proteins, and sugars) could serve as nutrients to live microorganisms, leading to their enhanced cell growth and biofilm formation. To test this hypothesis, we complemented the media with different concentrations of dead cells as described below. The comparison of total cell growth and biofilm formation in the presence of dead cells showed that higher concentrations of dead cells led to enhancement of bacterial growth and biofilm formation (Figure 2). This data suggests that live cells can utilize dead cells as a carbon source for growth and thus enhance biofilm formation.

The enhancement of biomass observed in the presence of low to moderate SWNT concentrations (5 to 300 mg/L) (Figure 1) was similar to the biomass enhancement in the presence of dead cells (Figure 2). For instance, the biofilm biomass formed in the presence of 100 mg/L of SWNTs had an optical density of 0.25 (Figure 1b), and the biofilm biomass formed with the media supplemented with 5×10^7 CFU/mL of dead cells (dilution of 10^{-1}) had the same optical density (Figure 2b), as opposed to optical densities of 0.15 in both control experiments (no dead cells and no SWNTs). Thus, the enhanced biofilm biomass in the presence of SWNTs can be explained by the release of intracellular materials of dead or damaged cells, which can then be used as a carbon source by live microorganisms.

On the basis of these results, we propose the following mechanism for the impact of SWNTs on cell growth and biofilm formation when low to moderate concentrations of SNWTs are introduced into aquatic environments. SWNTs in aquatic environments are in a bundled and aggregated form. Upon direct contact with microbes, these aggregates will damage cell walls resulting in release of intracellular materials (*14, 16*). The intracellular materials can be used as nutrients and lead to enhancement of biofilm formation by live cells not in contact with SWNTs. At the same time, the cells in direct contact with SWNTs can serve as a protective barrier or as a support for live

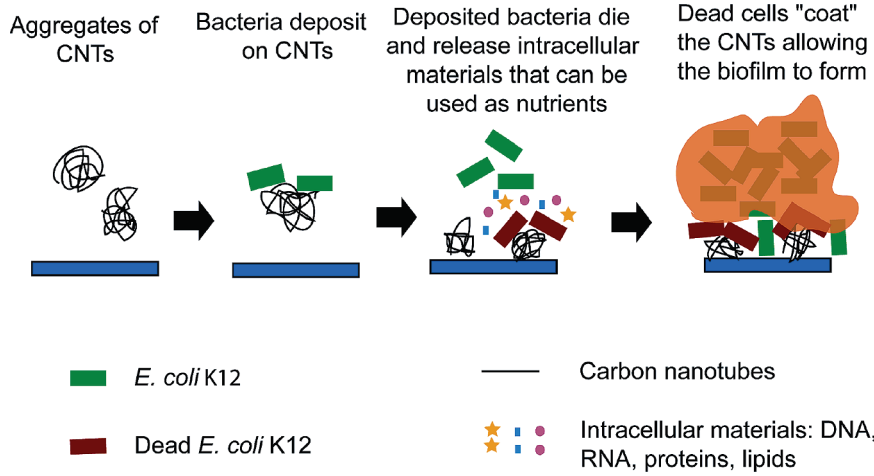

FIGURE 3. Proposed mechanism for the effects of SWNTs on cell growth and biofilm formation at low to moderate SNWT concentrations.

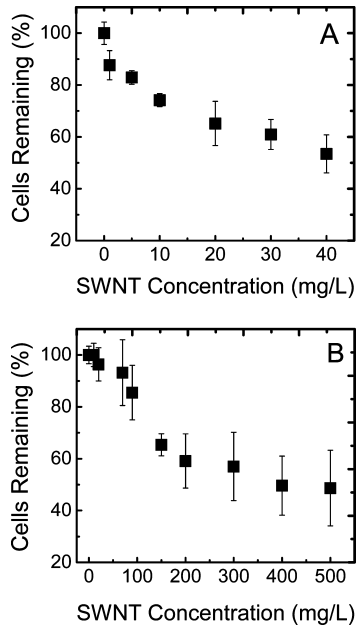

FIGURE 4. Impact of different SWNT concentrations on the release of *E. coli* K12 cells from mature biofilms in (A) absence or (B) presence of soluble EPS. Biofilms were allowed to form for 48 h under static conditions at 37 °C in M63 media supplemented with 1% D-mannose. After 48 h, the supernatant (unattached cells) was removed and only the attached biofilm was exposed for 1 h to different concentrations of SWNTs without (A) or with (B) the presence of soluble EPS. The biofilm biomass was quantified by OD_{540}, with 100% representing the biomass (OD_{540} reading) without exposure to SWNTs. Note the different range of SWNT concentrations in A and B. Each data point represents the percentage of cells remaining attached to the substratum. The results are based on measurements from 6 replicates. Error bars represent the standard deviation.

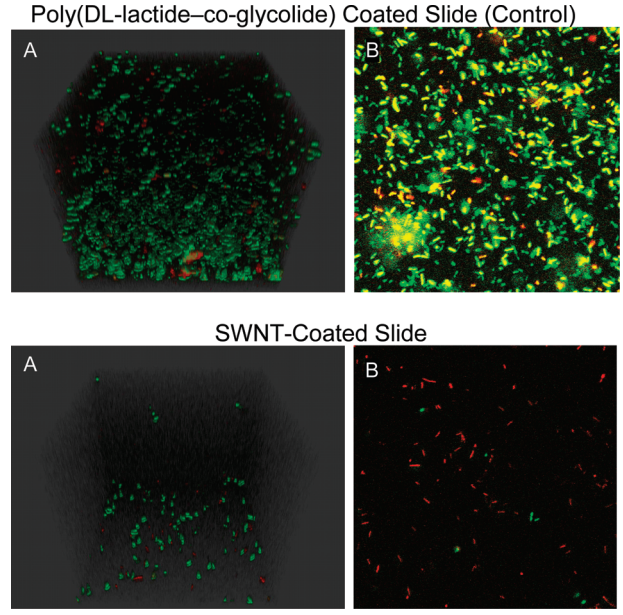

FIGURE 5. LSCM micrographs of biofilm attached to glass slide coated with SWNTs or poly (DL-lactide-co-glycolide) (control). Experiment was done under static conditions at 37 °C in M63 media supplemented with 1% D-mannose for 48 h. The three-dimensional reconstruction was done using ImageJ software (NIH) of LSCM images from planar images acquired at depth intervals of 2 μm. (A) Corresponds to cross section views of the biofilm growth on the different substrata. (B) Corresponds to bottom views of biofilms in direct contact with the substratum. The field view for each figure is a perspective of 112.5 μm × 112.5 μm × 104.4 μm (W × H × D). The green cells correspond to cells stained with SYTO9 (live cells) and the red cells correspond to cells stained with propidium iodide (dead cells).

cells, thus allowing the biofilm to form on top or in surrounding areas without SWNTs (Figure 3).

Influence of SWNT Addition to a Developed Biofilm with or without EPS. In fully mature biofilms in aquatic environments, the EPS secreted by the biofilm are likely to play an important role in SWNT toxicity. Several studies suggest that the EPS in biofilms interact with antimicrobials, such as antibiotics, and protect the cells either by preventing access of antibiotics to the biofilm or by effectively reducing their concentration (*27*). In addition to antibiotics, the EPS matrix may play a significant protective role against other antimicrobials, such as reactive oxygen species (*28*). In this case, the matrix acts as a reactive sink for these species (e.g., hydroxyl radicals and superoxide anions) and may constitute a significant diffusional barrier. The EPS matrix may also provide protection from toxic levels of heavy metals via binding of metals to various EPS functional groups.

Our results indicate that the soluble EPS produced by biofilms play an important role in mitigating the toxic effects of SWNTs. The results in Figure 4 show that biofilms without soluble EPS in the supernatant had significant loss of biomass because of cell detachment from the biofilm in the presence of much lower concentrations of SWNTs compared to biofilms containing soluble EPS. For the latter, we needed to use SWNT concentrations that were 10 times higher than the case without EPS to obtain similar cell loss from the biofilms. Our data indicates that soluble EPS play an important role as a protective barrier against the toxic effects of SWNTs, as observed with other antimicrobial agents.

The EPS in biofilms comprise proteins, DNA, RNA, and polysaccharides, with the majority being polysaccharides (*27*). In general, many of the polysaccharides present in biofilms are relatively soluble and produce highly viscous aqueous solutions because of their large molecular weight (*27*). The EPS produced by the *E. coli* biofilm is also highly viscous because of its composition (*29*). The viscous layer of the soluble EPS can reduce the transport of nanomaterials and hence reduce the contact of SWNTs with the cells in the biofilm, thereby making the SWNTs much less toxic to cells

TABLE 1. Confocal Microscopy Data for Biofilm Growth for 48 h in M63 Media with 1% D-Mannose on SWNT-Coated and Non-Coated (Control) Slides[a]

analyses	control	SWNT-coated
total biomass ($\mu m^3/\mu m^2$)	9.11 ± 3.91	0.12 ± 0.04
area occupied by cells (%)	30.89 ± 14.76	3.20 ± 0.13
average thickness (μm)	16.27 ± 10.19	0.098 ± 0.043
maximum thickness (μm)	66.3 ± 41.3	6.2 ± 2.5
roughness coefficient (range: zero-infinity)	0.577 ± 0.006	1.944 ± 0.035

[a] Results correspond to average of triplicate slides for each condition. Analyses were done using the COMSTAT program.

in mature biofilms than in other biofilm phases. Furthermore, it has been suggested that CNTs can produce reactive oxygen species that can act as an antimicrobial agent (*14*). In this case, the EPS can act as a sink for the reactive oxygen species (*28*).

Biofilm Development on Immobilized SWNTs. In the present study we investigated the structures of *E. coli* biofilms formed on surfaces coated with SWNTs and on control surfaces without SWNTs. For this purpose we used COMSTAT, a three-dimensional biofilm program, to analyze biofilm total biomass, substratum coverage, average thickness, maximum thickness, and roughness in confocal image stacks (*24*). These biofilm properties, summarized in Table 1, were selected because they can be interpreted in biological and physical terms.

Total biomass represents the volume of the biofilm per unit area, and also provides an estimate of the biofilm biomass attached to the substratum. The area occupied by cells represents the area coverage in the first layer of cells attached to the substratum (*24*). Substratum coverage reflects how efficiently the substratum is colonized by the microorganisms. In our experiments, the total biomass and the area occupied by the cells showed 10 times less biofilm colonization of the substratum and biofilm biomass production for the SWNT-coated substratum than the control (Table 1), indicating that SWNT-coated slides substantially inhibit bacterial colonization.

The average thickness provides a measure of the spatial size of the biofilm, while maximum thickness is defined as the maximum thickness over the image stacks, ignoring pores and voids inside the biofilm. Biofilm roughness provides a measure of how much the thickness of the biofilm varies, and also indicates biofilm heterogeneity (*24*). The average and maximum thickness values of the biofilms in our SWNT-coated slides were significantly lower than in the control slides, pointing out to the inhibiting effect of SWNTs on biofilm formation. SWNTs also impacted the biofilm roughness coefficient, resulting in a rougher and patchy bacterial colonization for the SWNT-coated slides. In fact, the average thickness data and the roughness coefficient (Table 1) indicate that the SWNT-coated surface was barely colonized, since the average thickness was much less than 1 μm, roughly the size of an *E. coli* bacterial cell. This conclusion that the majority of the SWNT-coated substratum did not contain any cells is also supported by analysis of the confocal images described below.

Besides the quantitative analyses of the LSCM image stacks conducted with COMSTAT (Table 1), we also compared the viability of cells in the biofilms formed on the control and SWNT-coated slides (Figure 5). In the control sample, the majority of the cells are alive (green), including the ones in direct contact with the substratum (top image, panel B). However, in stark contrast, *E. coli* on the SWNT-coated slides could hardly form any biofilm (bottom image, panel A). Additionally, the cells in direct contact with the substratum were mostly dead as indicated by the red color of the PI stain (bottom image, panel B). These findings clearly demonstrate that surfaces with deposited SWNTs significantly inhibit biofilm formation as a result of SWNT toxicity to cells in contact with the SWNT-covered surface.

Implications and Applications. The present study indicates that SWNTs are less toxic to mature biofilms than to bacterial cells in other biofilm phases. This finding is similar to previous observations with other antimicrobial compounds. We also observed that biofilm formation is markedly inhibited on surfaces coated with SWNTs. This finding has important implications for aquatic systems as engineered nanomaterials, such as SWNTs, will likely aggregate and end up in sediments. In addition to environmental implications, the inhibition of biofilm development on SWNT-coated surfaces suggests that SWNTs can be used as antimicrobial coatings in a variety of industrial and biomedical settings. These potential new applications, however, need to be thoroughly evaluated for their life cycle and impact on the environment. We also note that natural and engineered aquatic systems are more complex than the simplified system used in our study in terms of solution chemistry, presence of suspended particles, and natural organic matter, SWNT aggregation, and interaction of SWNTs with a diverse microbial community rather than bacterial monocultures. These factors, however, are beyond the scope of this paper and should be addressed in future investigations.

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